USE OF LIVE BACTERIA FOR GROWTH PROMOTION IN ANIMALS

FIELD OF THE INVENTION

The present invention relates to the field of growth promotion in animals. More specifically, the present invention relates to the use of a non-pathogenic *Escherichia coli* strain expressing the F4 (or K88) attachment factor, to either promote growth in animals or homogenize growth among a herd of animals.

BACKGROUND OF THE INVENTION

Growth promotion is a crucial issue for farm and breeding specialists, who mainly seek to optimize the production of healthy animals before slaughter or for research purposes. Such a concern should lead them to use growth promoting products that would prove beneficial to the animals and also to humans, in the case of meat-producing animals.

One major caveat in farms and breeding environments is the weight loss, slow growth rate along with a recrudescence of concomitant diseases, drug cost, and mortality which lead to a decrease in animal yields and ultimately to considerable economic losses. In this connection, post-weaning or post-hatching animals are particularly vulnerable to agents impeding growth.

Infections caused by either non-hygienic conditions or close proximity between animals, for example, are among the most common factors leading to the above-mentioned caveat.

One conventional solution used to alleviate this problem has been to use antibiotic growth promoters in feeds. However, use of antibiotic growth promoters is also highly controversial because, as is well known, even at sub-therapeutic doses, continued antibiotic use can lead to selection of antibiotic-resistant bacterial strains in the treated animals (Arnold S *et al.*; Wegener HC *et*

A further object of the present invention is to provide a method of promoting growth in an animal, said method comprising the step of feeding said animal with an effective amount of an F4+ non-pathogenic *Escherichia coli* strain.

Yet another object of the present invention is to provide a method of homogenizing growth among a herd of animals, said method comprising the step of feeding said animals with an effective amount of an F4+ non-pathogenic *Escherichia coli* strain.

Owing to the use of $F4^+$ non-pathogenic *Escherichia coli* strains, the invention finds an advantage in situations wherein rapid growth promotion and growth homogenization of an animal are particularly needed. For instance, use of $F4^+$ non-pathogenic *Escherichia coli* strains in animals reared for meat production allows to bring these animals to market weight or slaughter weight in a shorter growing period than that of their untreated counterparts.

The present invention may further find an advantage for growth promotion and growth homogenization of laboratory animals, such as rats and mice. As can be appreciated, bringing these laboratory animals to a given weight faster and more homogeneously preferably provides for more homogeneous and readily available samples of animals.

Another advantage of the present invention is that there is no recourse to antibiotic use to promote growth in animals. Therefore, problems such as development of antibiotic-resistant bacterial strains or allergies to antibiotics which particularly affect post-weaning animals, are alleviated.

Moreover, since heavy metals such as zinc oxide are not added to the animal's feed, contamination of the soil is also avoided. In other words, the present invention also provides for environment-friendly uses and methods.

The improved efficiency of feed conversion attained by the present method enables treated animals to reach any desired weight while consuming less food than untreated animals grown to the same weight. Moreover, while practicing the

Both methods comprise the step of feeding the animal(s) with an effective amount of an $F4^+$ non-pathogenic *Escherichia coli* strain.

The strain used in the present invention is characterized in that it expresses the attachment factor F4, while being non-pathogenic. According to a preferred aspect of the invention, the $F4^+$ non-pathogenic *Escherichia coli* strain is selected from the group consisting of coliPROtec (Accession number IDAC 210105-01), JG1329, M226, P03-7586(175) or pMK005. More preferably, the present invention contemplates using the coliPROtec strain and/or mutants or variants thereof (see Example I for more details).

As used herein, the terms "mutants" and "variants of the coliPROtec strain are used for strains that have all the identifying characteristics of the coliPROtec strain, as provided in Example I. Mutant or variant strains may be identified as having a genome or part thereof that hybridizes under conditions of high stringency to the genome of the coliPROtec strain.

According to the above-mentioned aspects of the invention, the above-described strain is used in an "effective amount". By the expression "effective amount", it will be understood that the amount of an $F4^+$ non-pathogenic *Escherichia coli* strain is the amount that will elicit the biological response of a tissue, system or animal that is being sought by the researcher or the veterinarian, for example. In other words, such an effective amount of an $F4^+$ non-pathogenic *Escherichia coli* strain is the amount that is sufficient for promoting growth in an animal as well as homogenizing growth among a herd of animals.

It will be understood that a preferred effective amount of the strain contemplated by the present invention is at least about 5^E7 colony-forming units (CFU), and more preferably range from about 5^E7 to about 5^E9 CFU of the strain of interest per animal. By "about", it is meant that the CFU value of said strain can vary within a certain range depending on the margin of error of the method used to evaluate the number of CFU for such a strain.

According to a preferred aspect of the invention, the post-weaning animal is preferably a pig, preferably aged from about 10 to about 28 days old, at the onset of the trials. The pig of interest is more preferably 17 days old.

According to another preferred aspect of the invention, the post-weaning animal is preferably a mouse, preferably aged from about 18 to about 28 days old, at the onset of the trials. The mouse of interest is more preferably 21 days old.

According to yet another preferred aspect of the invention, the post-hatching animal is preferably a chicken, preferably aged from about 1 to about 7 days old, at the onset of the trials. The chicken of interest is more preferably 1 day old. It will be understood that the expression "about 1 day old" means 24 hours or less after birth or hatching.

According to a preferred aspect of the present invention, the effective amount that can be given to pigs preferably varies from 5^E7 to 5^E9 CFU/ pig and is more preferably about 1^E9 CFU/pig.

According to another preferred aspect, the effective amount that can be given to chickens varies from about 5^E7 to 5^E9 CFU/chicken, and is more preferably 5^E8 CFU/chicken.

According to yet another preferred aspect, the effective amount that can be given to mice varies from about 5^E7 to 5^E9 CFU/mouse, and is more preferably 5^E8 CFU/mouse.

In the particular context of the methods of the invention, the effective amount of the strain may be fed to the animal as a single dosage or may be given according to a regimen, whereby it is effective. By the term "feeding", it should be understood that an $F4^+$ non-pathogenic *Escherichia coli* strain of the invention is provided to the animal under treatment so that the strain eventually reaches the gastro-intestinal tract, and more preferably the intestines.

iv- Feed conversion ratio (FCR): feed intake per animal or per group of animals for a particular period / Weight gain for the same animal or group of animals for the same particular period.

Advantageously, the strain of the invention may be used alone or in association with a feed acceptable carrier. As used herein, the expression "feed acceptable carrier" refers to any carrier, diluent or excipient that is compatible with the strain of the invention and can be given to an animal without adverse effects. Suitable feed acceptable carriers known in the art include, but are not limited to, water, saline, glucose, dextrose, or buffered solutions. Such a carrier is advantageously non-toxic to the strain and not harmful to the animal. It may also be biodegradable. A person skilled in the art will know how to select suitable carriers, such as carriers that are not harmful to the environment. Preferably also, this carrier is a suitable solid or liquid feed acceptable carrier.

A suitable solid feed acceptable carrier is a non-toxic ingestable carrier. For instance, this solid feed acceptable carrier may be a common solid feedstuff such as the component of a typical animal diet consisting of cereal products, such as barley meal, maize meal or wheat feed, nut and seed products, such as decorticated ground nut cake or cotton seed cake, or extracted cotton seed cake, together with minor amounts of, for example, feather meal, seaweed meal, bone meal, bone flour, chalk, salt, urea and vitamins; or it may be an inert solid diluent or carrier of no nutritional value, for example kaolin, talc, calcium carbonate, fuller's earth, attapulgus clay, ground oyster shells or ground limestone; or it may be starch or lactose.

A suitable liquid feed acceptable carrier is, for example, water and preferably drinking water; milk such as whole or skim milk; or a culture medium such as a trypsone-soy broth (TSB).

The following examples illustrate the wide range of potential applications of the present invention and are not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the

approximately 15 passages from the pig. This freeze-dried culture was used to produce the Master Seed of coliPROTec.

3. Biochemical analysis

An identification of the strain was done using the API system. The identification code 5544572 obtained referred as an *Escherichia coli* strain.

4. Virotyping

Virotyping of the coliPROtec strain was done by colony hybridization and/or polymerase chain reaction (PCR). Virotyping results showed that the coliPROtec strain was positive for F4 whereas it was negative for the following toxins:

LT, STa, STb, STaH, Stx1, Stx2, VT2vp1, VT2vh, Aero, Tsh, CDT3, CDT4, CNF1, CNF2, HlyA, HlyC, Ehx, East1.

Furthermore, the coliPROtec strain was also negative for the following adhesins or putative adhesins:

F5, F6, F18, F41, F17, P fimbriae, AIDA, AFA, SFA, CS31a, daaE, Paa, aggR, ARF/1, Eae, CFAI, CFAII(CS1coo), CFAII(CS3cst).

5. DNA fingerprinting

The coliPROtec strain was characterized using pulse-field gel electrophoresis (Figure 1).

Particular clones of the strain, for which the F4 fimbriae expression was stable after fermentation, were selected following repeated *in vitro* passages.

6. Summary Table and Data Sheet

The strains used in the following experiments are described in detail in the following Table 1 and Data Sheet.

EXAMPLE II: WEANED PIGS

Effects of F4$^+$ non-pathogenic *Escherichia coli* strains, in oral form, on the growth of pigs

A- Effect of coliPROtec on weight gain in weaned pigs

1. *Escherichia coli* strain:

The live *Escherichia coli* F4$^+$ non-pathogenic strain coliPROtec suspended in TSB was orally fed to weaned pigs. This strain is described in Example I.

2. Experiments

2.1 Animals

Five (5) trials were performed for a total of 45 treated and 45 untreated pigs. These pigs came from different commercial farms.

2.2 Trials

The trials were conducted at the Faculté de médicine vétérinaire, Université de Montréal, under the following schedule.

2.2.1 Trial groups

GROUP NO. (n)	DESCRIPTION
1(45)	Untreated
2(45)	Treated with coliProtec (with 5^E9/pig)

Table 3 : Daily weight gain

Days	Daily weight gain (g)		T-test	Group 2 vs Group 1 (g)
	Group 1	Group 2		
5 to 20	405	458	$p = 0.007$	+53

4. Analysis and Conclusion

At day 5 post-weaning, giving coliPROtec, the weight of the Groups 1 and 2 (untreated and treated with coliPROtec) was not statistically different. However, at day 20 post-weaning (15 days after the treatment), the treated animals were 849g heavier and demonstrated a daily weight gain of 53g more than untreated animals. These differences were statistically significant.

Of note, the conventional antibiotic growth promoters generally increase the weight gain by 3,3 to 8,8% (Doyle, M.E., Food Research Institute, University of Wisconsin, 2001). As demonstrated here, the coliPROtec strain increased the daily weight gain by 11% during the 2 weeks following the single dose.

B- Effects of live F4$^+$ non pathogenic *Escherichia coli* strains, in oral form, on the growth of weaned pigs

1. *Escherichia coli* strains

The strains used are described in Example I.

2. Experiments

2.1. Animals

Forty nine (49) 17-day-old weaned pigs, originating from a clean, conventional pig farm, were used in the present experiments. These piglets had a body weight of 5 ± 1 kg.

2.2. Trials

2.2.3 Evaluated parameters

During the trial, pigs had ad libitum access to feed and water. They were observed twice daily for general health and presence of diarrhea. From day 0 to day 14, pigs were fed with a commercial starter feed containing 23% protein without addition of zinc oxide or antibiotics. For the last 3 days, the feed contained 19% protein.

3. Results

3.1 $F4^+$ *Escherichia coli* and pathogenic ETEC status of pigs

At the beginning of the trial, thus before treatment, some pigs from all groups were colonized by an $F4^+$ strain possessing the toxin STb (Table 4). All pigs of the control group (Group 1) and of the group treated with an $F4^-$ *Escherichia coli* strain (Group 2), were colonized by this F4:STb strain at day 9 (Table 5). Although this strain (O45:F4:STb) is not a usual ETEC strain causing post-weaning diarrhea in pigs, we can not exclude that it is pathogenic. However, no animal demonstrated clinical signs associated with post-weaning diarrhea during the trial.

Since both control groups were colonized by this $F4^+$ strain, it is difficult to evaluate the effect of the tested $F4^+$ strains on animal growth performance.

Table 4: Identification of the status of pigs for excretion of $F4^+$ *Escherichia coli* strain before treatment (Day 1; PCR analysis on feces)

	Number of pigs with feces positive for F4	Other virulence factors identified
Group 1[*1]	1	LT and STb
Group 2	1	STb
Group 3	2	LT and STb
Group 4	1	LT and STb
Group 5	1	LT and STb
Group 6	3	LT and STb
Group 7	1	STb

*1: Treatment: Group 1; control, Group 2; F4-negative *Escherichia coli* strain at days 1 and 4, Groups 3 to 7; $F4^+$ strains at days 1 and 4.

Nevertheless, Groups 3 and 7, treated with the JFF4 and the recombinant strains, respectively, had a higher weight at days 4 and 9, but weights were subsequently more similar between groups (Table 6). During the first period of the trial (days 0 to 4), groups 3 and 7 had a percentage of weight gain of 18 and 15%, respectively, compared to 11% for both control groups (Groups 1 and 2; Figure 2). The low weight gain observed for the group 4 during this first period was attributed to 3 pigs that lost weight.

During the second period (days 4 to 9), groups 2, 4, 6 and 7 had a higher percentage of weight gain than that of the untreated group (group 1). The percentage of weight gain was more homogeneous during the third period (days 9 to 14) and was higher for groups 2 and 3 than for group 1, during the last period.

EXAMPLE III: BROILER CHICKENS

Effects of live F4$^+$ non pathogenic *Escherichia coli* strains, in oral form, on the growth of broiler chickens

1. *Escherichia coli* strains

The strains used are described in Example I.

2. Experiments

2.1 Animals

Sixty-three (63) male 1-day-old Cobbs broiler chicks, originating from a clean, conventional chicken farm. After hatching, chicks were transferred into animal facilities.

2.2 Trials

2.2.1 Trial groups

At hatching, the chickens were transferred into the animal facilities, containment rooms, Faculté de médecine vétérinaire, Saint-Hyacinthe, Quebec, Canada.

2.2.3 Evaluated parameters

Mean body weight (MBW), Mean daily weight gain (DWG), feed intake (FI), and feed conversion ratio (FCR) were all evaluated in the present assays.

During the trial, chickens had *ad libitum* access to feed and water. They were observed twice daily for the general health and presence of diarrhea. From day 0 to day 24, chickens received a standard commercial feed for chicks (without antibiotics). From day 24 to day 28, they received a standard development commercial feed (without antibiotics).

3. Results

3.1 $F4^+$ *Escherichia coli* and pathogenic ETEC status of chickens

No fecal sample was positive for F4, STa, STb or LT at day 0, before treatment. Fecal excretion of $F4^+$ *Escherichia coli* was detected in treated groups (groups 3 to 7) at days 2 and 4, but not subsequently.

3.2 General health and diarrhea assessment

Animals were in good health during the trial and no diarrhea was observed. Three (3) animals of group 7 were euthanized at day 23 or 25 due to their deteriorating general health status. No gastro-intestinal clinical sign was observed in these chickens. Necropsy reports on these chickens from the Pathology department (Faculté de médecine vétérinaire, Saint-Hyacinthe, Quebec, Canada) revealed that these chickens died from the ascites syndrome, a frequent non infectious disease, generally associated with cardiac insufficiency, in broiler chickens.

The DWG of all groups treated with $F4^+$ strains was higher than that of both control groups during the days following the treatments (days 4 to 9) and during the fourth period (days 14 to 18) for group 5 (Table 8).

F4-negative strain:

The MBW and DWG were similar or lower for the group treated with the F4-negative strain than for the untreated group.

A linear model with repeated measures, using the day as within-subject factor and the group as between-subject factor, showed no effect of treatment on growth of chickens (p = 0.97). *Post hoc* analysis was done to check for differences between each treated group (groups 2 to 7) and the untreated group (group 1) on each day. Weight was not significantly different between each treated group and the untreated group on each day. Differences observed in the descriptive analysis were not significant probably due to a higher variability than expected for the weight of chickens in each group, in particular for the untreated group.

3.4 Feed intake (FI)

Table 9: Feed intake (24-hr-period) of chickens after treatment with $F4^+$ *Escherichia coli* strains

	Feed intake (g) of groups (per animal) for 24-hour-periods			
Group	Day 2	Day 9	Day 17	Day 24
1[*1]	144 (16,0)	576 (64,0)	1043 (115,9)	1316 (146,2)
2	146 (16,2)	522 (58,0)	951 (105,7)	1180 (131,1)
3	128 (14,2)	570 (63,3)	915 (101,7)	1305 (145,0)
4	136 (15,1)	498 (55,3)	880 (97,8)	1389 (154,3)
5	169 (18,8)	526 (58,4)	976 (108,4)	1274 (141,6)
6	145 (16,1)	632 (70,2)	923 (102,6)	1535 (170,6)
7	144 (16,0)	695 (77,2)	1013 (112,6)	1006[*2] (143,7)

1: Treatment: Group 1; control, Group 2; F4-negative *Escherichia coli* strain at days 1 and 4, Groups 3 to 7; F4-positive strains at days 1 and 4.
[*2]: Two euthanized animals (n=7 instead of 9)

Other F4$^+$ strains: The FCR was lower than that of both control groups (groups 1 and 2) for group 7 (day 2), groups 3 and 4 (days 9 and 17), and groups 3 and 6 (day 17; table 4)

F4-negative strain: The FCR of the group treated with an F4-negative strain was lower than that of the untreated group (group 1), at day 17 only.

A linear model showed no effect of treatment on the feed conversion ratio of chickens (p = 0.68).

4. Analysis and Conclusion

Results demonstrate that F4$^+$ non-pathogenic *E. coli* strains, including the JFF4 strain, increase the growth performance of chickens. The growth performance was positively affected, especially for the days immediately following the second treatment, the DWG being higher for all treated groups than for the untreated group, during days 4 to 9. Two F4$^+$ strains, including the JFF4 strain, also had a higher DWG than both control groups during days 14 to 18. The improvement in the growth performance during the days immediately following treatments (days 4 to 9) resulted in a higher weight for groups treated with F4$^+$ strains than for both control groups until day 18 or, for some groups, day 23.

The higher DWG observed during the short period post-treatment affected positively the MBW of treated groups until days 18 or 23, depending on the strain. Greater weight of treated groups was not associated with higher feed intake. Furthermore, FI was sometimes lower for the treated groups than for the untreated group, thus lowering the feed conversion ratio.

This effect on growth performance is associated with the F4 determinant or with strains expressing the F4 determinant since the group treated with the F4-negative *Escherichia coli* strain did not show this effect and had similar growth performance to the untreated group.

2.2.2 Trial schedule

DAY NO.	DESCRIPTION
0	Arrival of the 21 day-old weaned mice at the animal facilities; identification, weighing and grouping of mice; for each treated group, 5 males and 5 females were grouped in 2 cages. Sampling of feces for evaluation of the excretion of F4, LT, STa and/or STb positive *Escherichia coli* (PCR)
1and 4	Approximately 1E9 CFU of *Escherichia coli* bacteria in trypsone broth (TSB) per mice given orally using an oesophageal needle. The control group received TSB only.
1, 4, 9, 14, 18, 23 and 28	Weighing of the animals; evaluation of the feed consumption for the period from the day of the previous weighing to the day of the weighing. Feces were sampled for evaluation of excretion of F4, LT, STa and/or STb positive *Escherichia coli* (PCR)
28	Euthanasia of animals

Variables evaluated: Mean body weight gain (MBW), feed intake (FI), and feed conversion ratio (FCR).

During the trial, mice had *ad libitum* access to feed and water. They were observed twice daily for general health and presence of diarrhea.

3. Results

3.1. $F4^+$ *Escherichia coli* and pathogenic ETEC status of mice

No fecal sample was positive for F4, STa, STb or LT at day 0. F4 was identified in the feces of groups 3, 4, 5 and 7 on the day after the first and/or up to 10 days after the second treatment with the $F4^+$ strains. No STa, STb or LT was detected at any time during the experiment.

3.2. General health and diarrhea assessment

All animals were in good health during the trial and no diarrhea was observed.

Strain JFF4: The FI of group 3 (JFF4), was lower than that of both the untreated group (group 1) and the group treated with the F4-negative strain (group 2) between days 4 to 9, early after treatment. However, it was lower than that of the untreated group but similar to that of the group treated with the F4-negative strain for most of the following periods, except days 14 to 18 (Table 12).

Other F4$^+$ strains: The FI of all other F4$^+$ strains was lower than that of both control groups (groups 1 and 2) during the period days 4 to 9, early after the treatment (Table 12). Subsequently, the FI varied, depending on the strain (Table 12).

F4-negative strain: The FI of the group treated with an F4-negative strain (group 2) was similar to that observed for the untreated group (group 1) until day 9, and was subsequently lower than the latter. Thus, the reduction of the FI for group 2 was observed later after treatment than for groups treated with F4$^+$ strains.

The linear model showed a significant effect of the treatment on feed intake ($p < 0.0001$). *Post-hoc* Dunnett's test showed that the feed consumption was significantly higher for the control group (Group 1) than for the groups 2, 3, 4, and 7, treated with the 862, JFF4, JG1329, and pmK005 strains, respectively (Table 12). For these groups, mice ate less feed to reach the same weight.

3.5 Feed conversion ratio (FCR)

Table 13: Feed intake of mice after treatment with F4$^+$ *Escherichia coli* strains

	Feed conversion ratio of groups	
Group	Days 0-14	Days 14-28
1[*1]	7,30	18,39
2	6,50	14,96
3	[redacted]	18,34
4	6,62	17,11
5	7,40	14,17
6	7,04	17,65
7	8,44	13,15

[*1]: Treatment: Group 1; control, Group 2; F4-negative *Escherichia coli* strain at days 1 and 4, Groups 3 to 7; F4$^+$ strains at days 1 and 4.

REFERENCES

Arnold S., Gassner B., Giger T. and Zwahlen R. Banning antimicrobial growth promoters in feedstuffs does not result in increased therapeutic use of antibiotics in medicated feed in pig farming. *Pharmacoepidemiology and Drug Safety* 2004; 13: 323-331.

Wegener HC, Aarestrup FM, Bogo Jensen L, Hammerum AM and Bager F. Use of antimicrobial growth promoters in food animals and Enterococcus faecium resistance to therapeutic antimicrobial drugs in Europe. *Emerging Infectious Diseases* 1999; 5 (3): 329- 335.

Schwarz S, Kehrenberg C and Walsh TR. Use of antimicrobial agents in veterinary medicine and food animal production. *International Journal of Antimicrobial Agents* 2001; 17: 431-437.

Bernardeau M, Vernoux JP and Gueguen M. Safety and efficacy of probiotic lactobacilli in promoting growth in post-weaning Swiss mice. *International Journal of Food Microbiology* 2002; 77(1-2): 19-27.